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Research on the Selection of Al Tolerant Alfalfa(*Medicago sativa* L.) on the Somatic Cell Level

I. Effect of some factors affecting callus induction of alfalfa

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體細胞 水準에서의 耐 Al 性 알팔파의 選拔에 關한 硏究

I. 알팔파의 캘러스 誘導에 미치는 몇가지 要因의 影響

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摘 要

本實驗은 Vernal 알팔파의 callus 誘導에 미치는 몇가지 要因들에 對하여 糾明하고자 遂行되었으며 얻어진 結果는 다음과 같다.

Callus 誘導에서 auxin 源으로서 2,4-D를 2-5mg/l 單用處理한 것이 가장 좋았으며, 여러가지 基本培地 中에서는 B5와 SH가 callus 誘導에 가장 效果的이었는데, PC와 MS 培地에서 生成된 callus는 friable 하였다.

培地의 pH는 5.8이 가장 좋았으며 7.0 以上에서는 callus는 거의 생장하지 않았다. 組織片은 9日齡된 植物體의 것이 가장 좋았으며, 이들 前後하여 callus의 生成量은 減少하였다. Callus 誘導時, 光條件의 有無는 큰 影響을 나타내지 않았으나, 빛 條件에서는 callus 組織은 푸르게 되었다.

I. Introduction

Alfalfa(*Medicago sativa* L.) appears to have originated in the mountainous regions east of the Mediterranean in southwestern Asia. It is one of the most widely cultivated forages in America because of its good palatability, high feeding value, good nitrogen fixation capacity, and resistance to drought and heat (Smith, 1981).

Alfalfa was introduced into Korea as late as in the 1960's, and moreover its spreading was even slower in spite of its excellent quality because of soil acidity of Korea (Jo, J., 1984).

Among leguminous forages, alfalfa is especially sensitive to soil acidity and grows scarcely in the soil pH of 5.0-5.5 (Jo, J. et al., 1980). In acidic soil, exchangeable aluminum is known to have more deleterious effect on the plant growth than other factors such as hydrogen ions and deficiency of essential elements.

Accordingly, this research was conducted to determine factors affecting induction of callus which is a potential material for the selection of Al-tolerant alfalfa on the somatic cell level.

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II. Materials and Methods

Plant materials

Seeds of Vernal alfalfa (*Medicago sativa* L.) were sterilized for 5 minutes in 1 % sodium hypochlorite, 3 minutes in 1 % hydrogen peroxide, and were washed

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thoroughly in sterile distilled water. They were germinated in the dark at 28°C on the SH basal medium solidified with 0.7 % agar and containing 2 % sucrose. The sterilized seeds were germinated in the dark for three days and were maintained under a 16 hr photoperiod of fluorescent light (1500 Lux) for 6 days.

Callus induction

Seedlings of 5-9 days old were dissected into cotyledons and 10 mm hypocotyl explants. Callus was induced in the SH medium supplemented with 4 mg/l 2,4-D alone as a growth regulator at 28°C for 3 or 4 weeks under the 16 hr photoperiod of flourescent light. All media were autoclaved at 121°C for 20 minutes.

After 3 or 4 weeks culture on the callus induction medium, callus fresh weight was measured and the callus was subcultured on the fresh medium with about 10 days intervals for preservation. The effect of growth regulators was compared among the combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid(NAA), and indole-3-acetic acid(IAA) as auxin sources and 6-benzyl aminopurine(BAP) and kinetin as cytokinin sources. Basal media were compared among B0, B5, MS, PC, and SH. Medium pH, explant age, and agar concentration were also investigated to decide the most suitable treatment for callus induction. All the treatments were replicated six times and the data were arranged statistically in this experiment.

III. Results and Discussion

Callus induction

The effect of auxin sources and concentrations of callus induction of Vernal alfalfa is shown in figure 1. After 20 days incubation on the SH medium, the highest callus induction was obtained in the treatment of 2-5mg/l 2,4-D from cotyledon and 2mg/l NAA from hypocotyl explants. Brown and Atanassov(1985) reported cotyledons had tendency to yield much more callus than hypocotyls. In the comparison among 2,4-D, NAA and IAA at 1.0mg/l, 2,4-D failed to stimulate more callus induction than the other auxin sources, but at concentrations above it, 2,4-D produced more

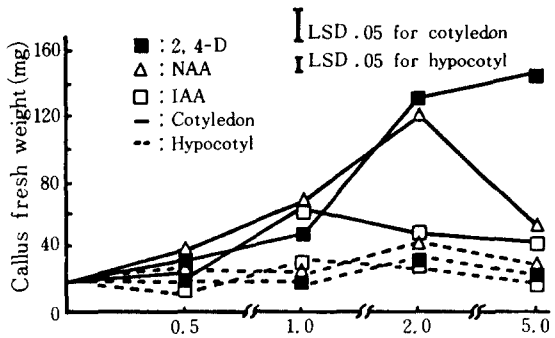

Fig. 1. Effect of auxin sources and concentrations on the callus induction of Vernal alfalfa in the SH basal medium

callus than NAA and IAA. Calli induced by 2,4-D were more friable than those induced by other auxin. Calli induced by NAA or IAA showed the development of root or shoot-like tissues during the incubation. There were significant differences in callus induction among auxin sources at the concentrations of above 2 mg/l.

Cytokinins were not necessarily required for the callus induction of alfalfa as shown in Table 1 though may scientists (Bingham & Saunders, 1975; Mitten et al., 1984; Brown & Atanassov, 1985) would add low concentrations of cytokinins. The more cytokinin added produced the less callus. In hypocotyl explants, addition of 0.1mg/l kinetin or BAP gave rise to the best callus production exceptionally.

There was a wide range of response in callus yield in both cotyledon and hypocotyl explants, that is, as much as 457mg from cotyledon explants at the concentration of 4 mg/l 2,4-D alone to as little as 48mg from hypocotyl explants at the concentration of 4mg/l 2,4-D + 2mg/l kinetin. In the treatment of 2mg/l 2,4-D + 2mg/l BAP, much callus was produced unexpectedly, which seemed to occur because of germplasm variation of alfalfa used.

Table 2 shows the effect of various media on the callus induction of Vernal alfalfa in two levels of 2,4-D. Callus yield was much higher in B5 or SH medium than in other media compared. This result was generally acceptable considering previous reports (Atanassov & Browns, 1984). In the relation between 2,4-D concentrations and media, the highest yield was obtained

Table 1. Effect of growth regulators on the callus induction of Vernal alfalfa in SH basal medium

Combination of growth regulator (mg/l)			Mean fresh weight of callus (mg)	
			Cotyledon	Hypocotyl
2, 4-D 2.0	Kinetin	0.0	375 abc*	119 bcd
		0.1	338 abcde	147 bcd
		0.5	291 abcdefg	101 bcd
		1.0	248 cdefg	74 bcd
		2.0	202 defg	55 cd
	BAP	0.1	353 abcd	138 bcd
		0.5	310 abcdef	130 bcd
		1.0	227 cdefg	120 bcd
		2.0	177 efg	328 a
2, 4-D 4.0	Kinetin	0.0	457 a	66 cd
		0.1	418 ab	151 bcd
		0.5	284 bcdefg	125 bcd
		1.0	224 cdefg	82 bcd
		2.0	171 fg	48 d
	BAP	0.1	299 abcdefg	188 b
		0.5	267 bcdefg	166 bc
		1.0	185 efg	70 cd
		2.0	136 g	68 cd

*Values followed by different letters are significantly different within column by Duncan's multiple range test (P 0.05)

with 4 mg/l 2,4-D in SH medium while the lowest yield was with 2 mg/l 2,4-D in SH medium among all the medium treatments. SH medium was especially favorable for long period culture over 60 days. The callus produced on PC or MS medium was more friable than that produced on other media. Atanassov and Brown (1984) reported that B5 medium was very effective for callus induction. But SH medium has been generally used in tissue culture of alfalfa and other legumes because it generally has higher regenerative capacity than other media (Shenk & Hildebrandt, 1972). The composition of nutrients and ion concentration of SH medium are similar to those of B5 medium.

The optimum pH was tested among 5 treatments of 5.0, 5.5, 5.8, 6.3 and 7.0. The highest callus yield was achieved at pH 5.8 ($P < 0.05$) in cotyledon explants (Figure 2) and it was correspondend well with general results (Bingham & Saunders, 1975; Schenk & Hildebrandt, 1972). The callus yield in hypocotyl explants

Table 2. Effect of various media on the callus induction of Vernal afalfa in two levels of 2, 4-D

2, 4-D conc. (mg/l)	Medium	Mean fresh weight of callus (mg)	
		Cotyledon	Hypocotyl
2.0	BO*	370 de**	79 d
	B_5	596 b	122 b
	MS	414 cd	120 b
	PC	383 de	86 cd
	SH	304 e	97 bcd
4.0	BO	333 de	105 bcd
	B_5	765 a	178 a
	MS	337 de	114 bc
	PC	341 de	85 cd
	SH	509 bc	115 bc

*BO : Blaydes, B_5 : Gamborg, MS : Murashige & Skoog, PC : Phillips & Collins, SH : Schenk & Hilderbrandt.
**Values followed by different letters are significantly different within column by Duncan's multiple range test (P 0.05).

was much lower in any pH treatment than that in cotyledon explants and no significant differences were detected among five treatments.

At the pH above 7.0, callus growth was quite inhibited and frequent browning was observed. Murashige & Skoog (1962) recommended adding agar and preheating the medium for a few minutes before pH adjustment because this avoided any appreciable pH change during autoclaving. They found the pH 5.7-5.8 was suitable for maintaining all the salts in the soluble form even with relatively high phosphate level. There were considerable differences between initial pH levels and levels following autoclaving particularly in the pH range of 5.7-8.5 in this experiment. Skirvin (1986) also stated that placing of explaints or callus tissues to the media accelerated the pH change regardless of original pH.

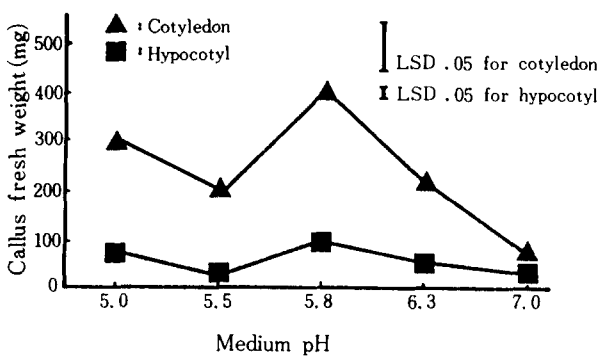

Fig. 2. Effect of medium pH on the callus induction of Vernal alfalfa

Callus induction was also influenced by the age of seedlings. Younger seedlings produced more callus than old ones as shown in Figure 3. There were significant differences among the ages of 5, 7, 9, 15 and 23 days old ($P < 0.05$). The seedling age for effective callus induction was around 9 days which was younger than that of Brown and Atanassov (1985) used. They utilized seedlings of two weeks old or more as explaints. George & Sherrington (1984) reported explants younger than 5 days old were not suitable for callus induction because of low cell density.

Figure 4 shows the effect of agar concentrations on the callus induction. The callus fresh weight was the highest in 0.5 % agar treatment. Agar concentrations

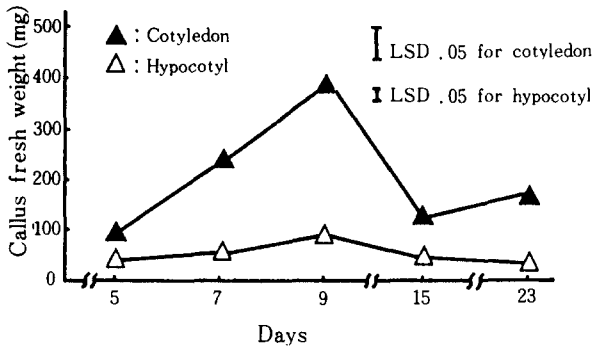

Fig. 3. Effect of explant age on the callus induction of Vernal alfalfa

more than 1 %, which brought about rapid browning of callus, was not suitable for callus production. On the contrary, the agar concentration less than 0.5 % had difficulty to handle and the treatments below 0.5 % were excluded in this experiment. The media containing 0.6 or 0.8 % agar resulted in white and wet calli while those containing higher levels brought dried and brown ones. George and Sherrington (1984) suggested that a reasonable cause of reduced growth by the addition of increased amount of agar might be the immobilization of the enzyme, invertase released from cultured tissues. Invertase immobilization would result in a reduced availability of glucose in the plant tissues.

Callus induction was not influenced by day length or illumination though data are not shown. Calli incubated under 16/8 hr light/dark cycle became more compact and green than those incubated under the dark.

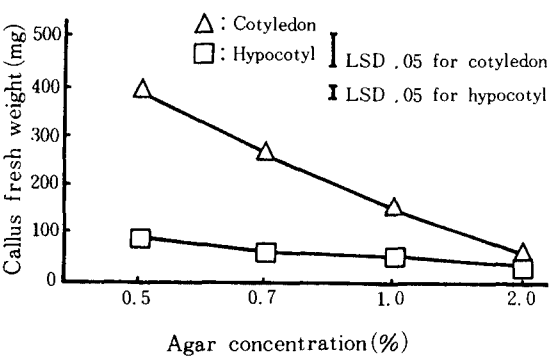

Fig. 4. Effect of agar concentration of the medium on the callus induction of Vernal alfalfa

Greening was caused by chlorophyll formation under the light, and it was thought to be account for the induction of compact calli. George and Sherrington (1984) reported chlorophyll synthesis was stimulated by red light. The wave length of the light used was not analysed in this experiment.

IV. Summary

This experiment was conducted to determine factors affecting callus induction of Vernal alfalfa. Growth regulators, basal medium, medium pH, explant age, and agar concentration for callus induction were investigated.

The results obtained were as follows: For the callus induction, 2-5 mg/l 2,4-D alone was found to be most effective on callus induction. Cytokinins did not have positive effect on the callus induction, and even the more cytokinin added induced the less callus.

Callus yield was much higher in B5 or SH medium than in any other media. The calli induced in PC and MS media were more friable than those induced in other media.

The medium pH of 5.8 gave the best response of callus induction. At higher than pH 7.0, callus induction was inhibited severely.

The effective seedling age for callus induction was around 9 days. In agar concentration, 0.5 % (W/V) was suitable for callus induction and it was severely depressed at above 1 %. Callus induction was not influenced by day length or illumination. Calli cultured under 16/8 hour light/dark cycle became more compact and green than those cultured under the dark.

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